LOCALIZATIONS OF ALKALINE AND ACID PHOSPHATASES IN THE EARLY EMBRYOGENESIS OF THE CHICK[1]

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Since Gomori's publication of a histochemical method for the demonstration of alkaline phosphatase, several studies of the distribution of alkaline phosphatase within tissues have appeared. Gomori himself (1941a) made a survey of the enzyme in a variety of adult mammalian organs; Kabat and Furth (1941) studied its occurrence in normal and tumorous tissues of mammals and chickens; Bourne (1943) investigated its presence in numerous tissues, giving special attention to the intra-cellular localizations; and Horowitz (1942) examined the interrelations of phosphatase and glycogen in fetal heads. Gomori (1941b) employed his somewhat less satisfactory technique for acid phosphatase in a study of acid phosphatase distribution in normal and tumorous tissues, and Wolf, Kabat and Newman (1943), after modifying the original technique to produce more regular results, used it in a study of acid phosphatases in the nervous system.

The present survey of the distribution of phosphatases in the early embryo of the chick was undertaken for two reasons. First it seemed likely that just as the studies cited above have aided in establishing correlations between phosphatase content and function in mature organs, so an embryological study might further elucidate the problem of the functional rôles of phosphatase by establishing correlations between phosphatase content and functional state in developing organs. Second, because of our increasing knowledge of the widespread importance of phosphate transfer in metabolism, it seemed worthwhile to examine the possibility that phosphatases play a part in the proliferative and differentiative processes of the early embryo.

Materials and Methods

These studies are based on White Leghorn embryos taken in stages from the unincubated blastoderm through the eighth day of incubation for all series of observations except those on the long bones of the hind limb, for which nine- and ten-day limbs were also used. At all stages, cold acetone was employed as fixative; after 2 to 24 hours in acetone, depending on size, the embryos were cleared in cedarwood oil and benzene, embedded in a paraffin-beeswax-bayberry wax mixture, and sectioned at 10 or 15 micra. Shrinkage of the cytoplasm occurred in many cells, but for gross histological examination chilled acetone proved an excellent fixative; for the preservation of alkaline as well as acid phosphatase it was superior to 85 per cent alcohol.

The histochemical method for the demonstration of alkaline phosphatase has been repeatedly described (Gomori, 1941a; Kabat and Furth, 1941; Bourne, 1943).

[1] Aided by a grant from the Rockefeller Foundation to Washington University.

The method was employed in these studies exactly as presented by Gomori, except that sodium glycerophosphate containing equal parts of the alpha and beta salts was used, since pure alpha glycerophosphate was not available; the pH was 9.3, and the time of incubation 1½ to 4 hours, for different specimens, at 38° C. Erythrosin was used as counterstain.

In the case of the acid phosphatase, Gomori's (1941b) formula for the incubating solution was altered to compensate for the precipitation of lead beta-glycerophosphate, as follows:

Acetate buffer at pH 4.8	2½	parts
5% lead nitrate	1	part
Distilled water	15	parts
2% Na glycerophosphate	3	parts

The ingredients should be mixed in the order given, and the solution be allowed to stand overnight in the refrigerator, since the precipitate develops slowly. The solution was filtered just before use, at which time any additional reagents were added. The pH is 5.1; the incubation time ranged from 5 to 16 hours, at 38°, since the concentration of acid phosphatase in most embryonic tissues is extremely low. In some cases at each stage 0.01 M $MnSO_4$ was added to the incubating solution in order to produce the stable black-staining precipitate described before (Moog, 1943b); and after it was discovered that ascorbic acid is a strong activator of acid phosphatase, series were also prepared with 0.01 M ascorbic acid added to the incubating solution. This phenomenon will be discussed in a later section.

After incubation the slides were rinsed in distilled water and dilute acetic acid, and stained by 2 minutes' exposure to light yellow ammonium sulfide, which results in the formation of brown or black lead sulfide. After thorough washing under the tap, the slides were dehydrated in the usual manner, counterstained with erythrosin, and mounted.

At all stages controls were made to check on the possible presence of preformed phosphates, by incubating slides in a solution from which glycerophosphate was omitted, and then staining them in the usual way. To a considerable extent also the alkaline and acid series served as controls for each other, since any tissue appearing negative to either enzyme could not contain preformed phosphate.

The stability of alkaline phosphatase in chick embryos has already been remarked on. The acid phosphatase is however troublesome to demonstrate with any regularity, so that material used for the study of this enzyme must be handled with great caution (cf. Moog, 1943a). The use of manganese improves the production of clearly visible precipitates in tissues low in acid phosphatase, but it does not control the considerable variations in activity found among individual embryos. Ascorbic acid, on the other hand, has proved valuable by reason of its ability to increase both the intensity and the uniformity of the normal deposits, especially in fresh preparations. Yet even with ascorbic acid variations continue to appear, and seem to reflect the extreme lability of the enzyme; for example, it will survive only a few days' storage in the refrigerator, even in paraffin, and it is quickly inactivated by temperatures above 60° C. The penetrating power of acetone was not a limiting factor, as in the tissues Wolf, Kabat and Newman (1943) used, for the deepest layers of the liver, even in eight-day embryos, gave more consistent results than some of the more superficial tissues.

Results

I. Alkaline Phosphatase

In the embryonic stages studied, alkaline phosphatase has been found present more generally in nuclei than in cytoplasm. On the basis of the relative concentration of the enzyme in nucleus and cytoplasm, in fact, three types of tissue can be described. (1) Those tissues, or cell-aggregations, in which both nuclei and cytoplasm stain intensely: most tissues show this condition during the first two or three days of development, or in some cases much longer, while in several tissues this condition is achieved in the course of differentiation. (2) Those tissues in which the nuclei are more reactive than the cytoplasm: this distribution is found in some regions very early, before the peak of phosphatase concentration is reached, and again it is found commonly later, when many tissues gradually lose their early reactivity as differentiation proceeds. A decrease in enzyme content always affects the cytoplasm before the nuclei. (3) Those tissues in which neither cytoplasm nor nuclei show marked phosphatase activity: in a few cases this condition obtains throughout the period studied, but more often the relatively phosphatase-free state results from the gradual disappearance of the enzyme from originally reactive cell groups during the course of differentiation. The nuclei are never found to be less reactive than the cytoplasm, nor do they ever become completely negative, although the cytoplasm frequently does so. In the least reactive nuclei, the nucleoli stain heavily and the nuclear membranes lightly. Where the phosphatase concentration is greater, the membranes stain more darkly, the karyoplasm takes on a deepening greyish coloration, and intensely stained masses make their appearance within the membrane; these masses are probably chromosomal detritus, since the chromosomes would be expected to disintegrate during several hours exposure to a solution at pH 9.3.[2] Finally, when the phosphatase concentration is very high, the nuclei stain solid black within a relatively short time. The usual high reactivity of the nuclei makes it sometimes difficult to decide whether a change in phosphatase concentration is real, or merely the apparent result of either lessened crowding of the nuclei or an increase in the amount of cytoplasm per nucleus. To rule out errors due to these factors, it was necessary to make careful comparisons of treated sections with others stained with ordinary nuclear stains.

In the descriptions which follow, the terms "positive" and "active" or "reactive" have been applied to tissues which show more than the minimal nuclear response described above (i.e., no deep stain except in the nucleoli). Those tissues which show only the minimal nuclear response are denoted "negative." An attempt has been made to rate both alkaline and acid phosphatase concentrations on a single scale on which 16 + represents a solid black deposit appearing after 1½ hours' incubation, and 1 + the lightest recognizable deposit appearing after 16 hours' incubation.

The first day

In the unincubated blastoderm both the incipient germ layers and their neighboring yolk cells contain alkaline phosphatase. The smaller, compact yolk granules (white yolk) clustered in the marginal zone are very strongly positive (12 +), the

[2] I am indebted to Dr. Jack Schultz for pointing this out to me.

larger granulated elements (yellow yolk) being much less so. The endoderm and ectoderm are about equally positive (9 +), but their reactivity is much greater in the heavily staining nuclei and in the enclosed yolk granules than in the cytoplasm. At the periphery of the blastodermal area the phosphatase content of the ectoderm wanes.

From the blastoderm sections it appeared that the white yolk is more rich in phosphatase than the yellow, which however seemed to contain a moderate amount of the enzyme also. But smears of yolk taken from regions away from the blastoderm revealed that neither type of yolk contains much phosphatase, although both take on a spurious stain due to the presence of preformed phosphates. The high activity of the white yolk around the embryonic area thus seems rather to be related to proximity to the embryonic tissue, than to be an intrinsic property of white yolk.

As the embryonic body itself is laid down toward the close of the first day, it has at the anterior end a phosphatase content as high as in the preceding tissues, or slightly higher in the case of the ectoderm; but there is a gradual decrease in all layers toward the posterior end. Thus a fairly uniform antero-posterior gradient in intensity of staining can be observed. As before, the reaction also decreases in the superficial ectoderm of the extra-embryonic area.

Two and three days

The brain, and the mesoderm at anterior levels, stain virtually solid black during the second day (Fig. 2). Posterior to the brain the ectoderm and mesoderm are slightly less reactive, and in the entoderm the nuclei stain only moderately, while the cytoplasm is almost negative. At the posterior tip of the body all tissues are still only slightly reactive (Fig. 3). Through the bulk of the body, however, the brain, nerve cord, mesoderm, endothelia, and also the notochord, contain considerable amounts of phosphatase (8 + to 12 +). The red blood cells are negative. The nuclei of the extra-embryonic membranes occasionally show more than

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PLATE I

1, 6—acid phosphatase
2–5, 7, 8—alkaline phosphatase

1. Through primitive groove. *ec*, ectoderm; *m*, mesoderm; *en*, endoderm; *v*, vitelline membrane.

2, 3. Anterior and posterior levels of a single 44-hour embryo; note greater concentration of phosphatase in ectoderm and mesoderm at anterior level. *h*, heart; *s*, somite; *lm*, lateral mesoderm.

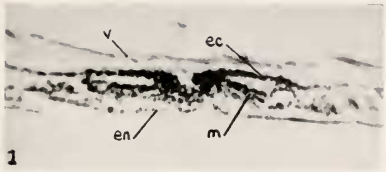
4. Through diencephalon of a 72-hour embryo; note greater phosphatase concentration in more ventral part of diencephalon.

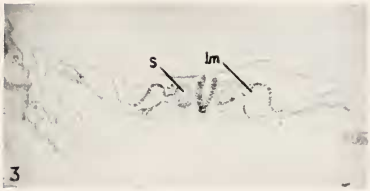
5. Through bulbus of a 5-day embryo; note high phosphatase content of valve and spongy lining tissue of bulbus. *m*, myocardium; *ct*, connective tissue; *a*, *a*, auricles.

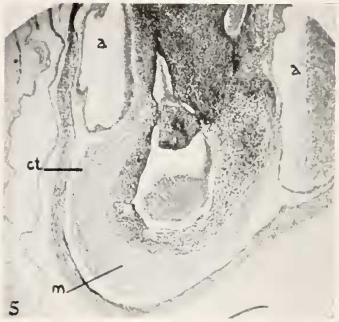
6. Through lung buds of a 5-day embryo. *a*, aorta; *b*, mesobronchus in lung bud; *o*, oesophagus; *l*, liver; *wd*, Wolffian duct.

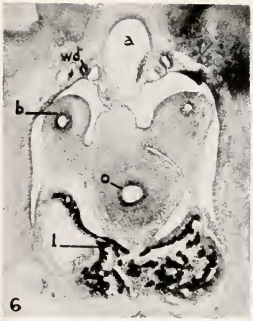
7. Differentiation of axial cartilage in fore-limb region of a 5-day embryo. *m*, phosphatase-containing membranous anlage of neural arch; *pc*, phosphatase-free protochondrium of future vertebral centrum; *n*, notochord; *a*, aorta. Note also phosphatase-free motor horns of spinal cord.

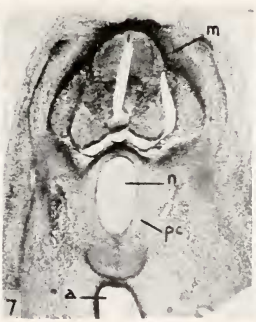
8. Liver diverticulum of 3-day embryo. *mg*, mid-gut; *l*, strongly positive liver diverticulum.











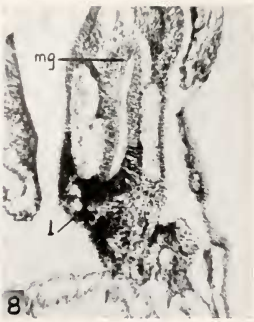


PLATE I

a minimal reaction, but more often they are negative so that there is usually a sharp line of demarcation between the embryonic and extra-embryonic tissues.

At 72 hours phosphatase is still distributed widely through the embryo, which continues to present a darkened appearance; but numerous tissues exhibit a lowered reactivity, evinced principally in the cytoplasm. This is especially true of the notochord at anterior levels, and the muscular wall of the heart.

The brain gives a strong phosphatase reaction in the most dorsal parts of the metencephalon and myelencephalon, but at about the level of the auditory capsule the hind brain is definitely less reactive (7 +) than the mid-brain, which is heavily blackened throughout. The infundibulum is moderately positive. The region of the diencephalon between the eyes appears much darker than the more dorsal region (Fig. 4), and this dorso-ventral gradation continues into the telencephalon. The dorsal part of the nerve cord and the neural crests react more strongly than the ventral part of the cord, but this difference disappears at more posterior levels, usually around the point where the amniotic folds are still open. The cranial ganglia show considerable deposits (10 +).

The eyes, the nasal placodes, and the outer faces of the auditory capsules are all strongly reactive (9 + to 10 +). The inner face of the capsule, and the lens, react weakly.

The muscular walls of the ventricle and atrium are negative, but the endothelial linings are positive. The bulbus gives a moderately strong reaction, while the walls of the ducti Cuvieri and the ventral aorta are blackened (9 +). This reactivity tends to persist in the aortic arches and dorsal aorta, but it fades in the latter as its walls become thinner toward the posterior end of the body. The cardinal veins show no particular reaction. The omphalomesenteric veins, where they enter the body, are lined with heavily reactive mesoderm, but this reactivity disappears as the vessels spread out over the yolk.

The pharynx wall is moderately positive, as is Rathke's pouch. The closely packed mesoderm in the visceral arches reacts to a lesser extent than the mesoderm of any other region. The thyroid is strongly positive. The laryngo-tracheal groove reacts noticeably, but the remainder of the alimentary canal still exhibits the low activity of the endoderm from which it is derived. The liver diverticula however are more positive than the tissue from which they arise (Fig. 8).

The mesoderm and loose mesenchyme are positive throughout the body, the re-

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PLATE II

9–15—alkaline phosphatase

9. Inner ear of 8-day embryo; note alternate light and dark staining patches of labyrinth wall. *c*, anterior semi-circular canal; *an*, acoustic ganglion; *my*, myelencephalon.

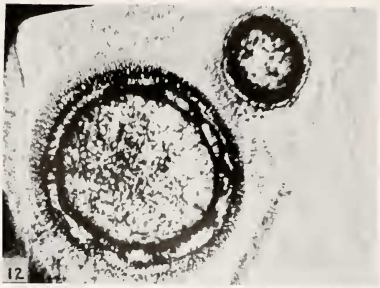
10. Optic chiasma of 8-day embryo. *oc*, central part of chiasma, just below optic nerves; *hy*, hypophysis.

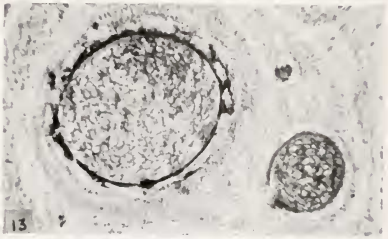
11. Longitudinal section through femur of 7-day embryo, showing thick coat of perichondral phosphatase in diaphysial region. (The dark band across the epiphysis is an artefact.)

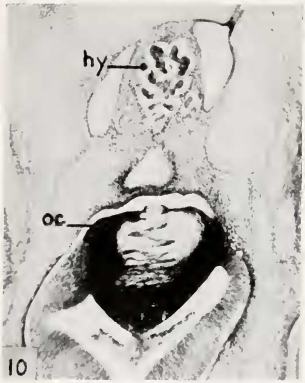
12. Transverse section through tibia and fibula of 8-day embryo. Note spongy nature of perichondral phosphatase layer, and also positive cells within the cartilage.

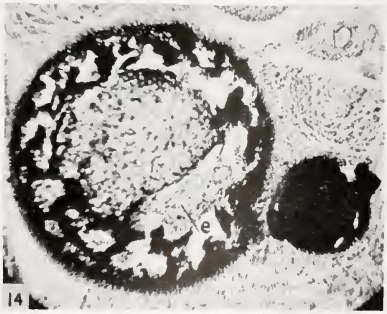
13. Control for 12 (opposite limb of same embryo). The stained material in the perichondral area is bone (calcium phosphate) deposited *in vivo*.

14, 15. As 12 and 13, for a 10-day embryo; section is near distal end of fibula. *e*, area of erosion.









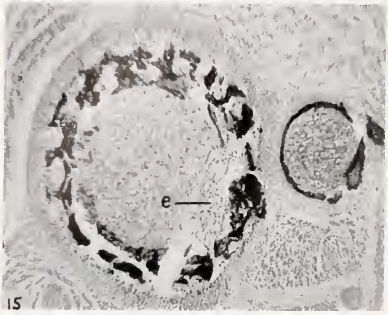


PLATE II

action still diminishing, however, toward the posterior end, as it does also in the neural tube. The nephric structures are darkened to the same extent as the surrounding mesenchyme.

Four to eight days

a. Nervous system

1. *The brain:*

Telencephalon.—The dorsal-ventral difference mentioned before is still evident in the cerebral hemispheres through the fifth day. On the ventral side there is a strongly positive region where the constriction between the hemispheres begins, but this disappears sharply where the partition is complete. Proceeding anteriorly one finds the outer and ventral faces on the hemispheres moderately positive (7 +), while the inner-dorsal faces are weakly so (4 +). The reaction gradually fades, so that the walls are almost negative near the olfactory part. On the sixth day the reactivity of the superior walls strengthens, and the phosphatase activity extends through the olfactory region.

On the seventh and eighth days the corpora striata and the pallium are lightly positive. A more intense reaction (7 +) appearing in the telencephalon medium however persists, somewhat attenuated, in the floor region of the hemispheres. The inner faces are virtually negative, as is the choroid plexus. The parencephalon is negative.

The epiphysis is negative when first formed, but phosphatase accumulates in the tubule cells as the glandular structure differentiates.

Diencephalon.—On the fourth day and later the ventral section of the posterior part of the diencephalon shows a strong reaction; but this diminishes just behind the eyes, so that for some distance between the eyes, as one proceeds anteriorly, only the central portion is strongly positive. The heavy stain at the entrance of the optic nerves, established earlier, persists; the nerves themselves are at first extremely reactive but their enzyme distribution becomes less uniform, for the differentiated supporting glia and connective tissue are negative, and only the nerve fibers retain the enzyme. As the thick walls of the thalamus develop, they show a reaction graded from moderate (5 +) dorsally to strong (9 +) on the floor. This

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PLATE III

19, 21—acid phosphatase
16–18, 20, 22, 23—alkaline phosphatase

16. Through neck of 8-day embryo. *t*, thyroid; *o*, oesophagus; *gn*, ganglion nodosum; *cc*, common carotid artery; *j*, jugular vein.

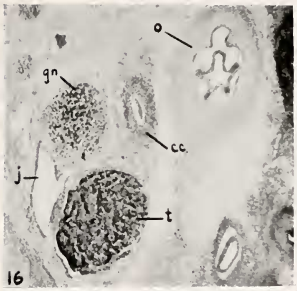
17. Part of myelencephalon of 7-day embryo. Note phosphatase-containing tissue around lumen, and sharp decrease in phosphatase content of axones from ganglion (*g*) as they enter myelencephalon.

18, 19. Proventriculus of 8-day embryo. Note phosphatase content of mesenchyme surrounding glands. *g*, gland; *cm*, circular muscle; *ca*, coeliac artery; *l*, liver. Note that with alkaline phosphatase liver cells are unstained and endothelium stained; with acid phosphatase cells are stained and endothelium unstained.

20, 21. Mesonephroi and associated structures of 8-day embryos. *gl*, glomerulus; *st*, secretory tubule; *ct*, collecting tubule; *md*, Müllerian duct; *wd*, Wolffian duct; *go*, gonad; *mt*, metanephric tubules and blastema; *r*, rib.

22. Blood vessel and positive endothelium in liver of 8-day embryo.

23. Positive endothelium in ventricle of 8-day embryo. *rc*, red cells.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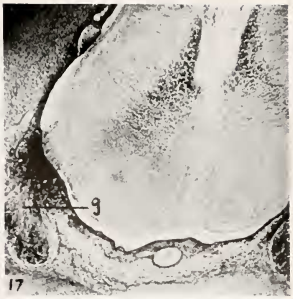
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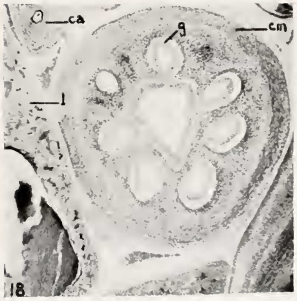
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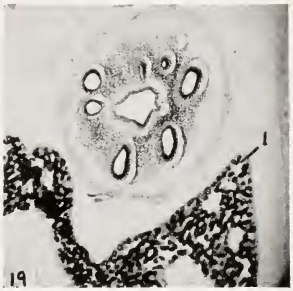
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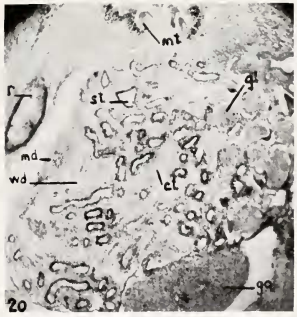
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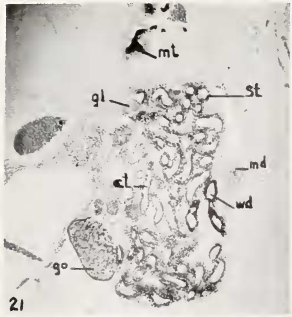
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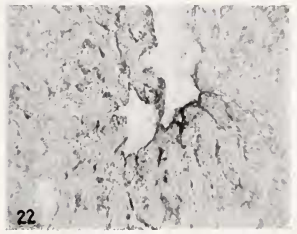
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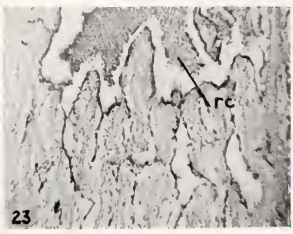
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PLATE III

intense reaction is continued around the lamina terminalis, and on the eighth day the optic chiasma (Fig. 10) shows extremely heavy deposits (12 +).

Mesencephalon.—A heavy deposit (8 +) in the outer wall of the more dorsal part of the optic lobes gives the slightly reactive inner tissue the appearance of being surrounded by a shell; with the persistence of this difference the outer region comes to include the layer of short radially arranged fibers that develop near the surface on the sixth day. The ventral and posterior walls of the aqueduct are markedly positive (7 +), the dorsal and anterior walls less so (4 +). This dorso-ventral gradient, as described above, continues forward into the diencephalon.

Metencephalon and myelencephalon.—At its junction with the mesencephalon, the metencephalon shares the high phosphatase concentration of the mesencephalic floor. On the fourth day this persists for a short distance posteriorly and then fades into a more moderate reaction which grows lighter on successive days. On the sixth day, however, heavy deposits (10 +) concentrate around the outer ventral corners of the fourth ventricle and taper off into the ventro-lateral tissue. This distribution continues backward, heavy deposits remaining close to the narrowing lumen while lighter deposits (7 +) gradually spread through the whole ventral region, except for the negative raphe (Fig. 17); as the spinal cord is entered, this pattern resolves itself into the cross-band of phosphatase-rich cells which has already been described (Moog, 1943a). The thin roof of the hind brain, and also the cerebellum, are negative.

2. *The cranial ganglia:*

Throughout the period under consideration all the cranial ganglia but the eighth show marked phosphatase activity (Fig. 16) of fairly uniform intensity (10 +). The axones connecting the ganglia to the brain are positive outside the brain and negative, or weakly positive, inside, the line of demarcation being extremely sharp (Fig. 17); the processes distal to the ganglia, however, are generally much less positive. Limited regions of the auditory ganglion stain darkly, but the bulk of the ganglion, including all those portions which amalgamate with auditory structures, are negative.

3. *The spinal cord:*

The spinal cord has already been reported on (Moog, 1943a). In terms of the scale used in this paper, the highest concentrations of alkaline phosphatase in the cord may be rated as 9 + to 11 +.

4. *The sense organs:*

The ear.—On the fourth day the auditory capsule, as before, exhibits alternate patches of lightly and darkly staining tissue; as development proceeds it becomes apparent that the lighter patches are the rudiments of the maculae and cristae (Fig. 9). These areas, like the branches of the auditory nerve that fuse with them, grow less reactive and on the eighth day are virtually negative. The rest of the labyrinth wall is strongly positive (10 +), as is the entire internal limiting membrane.

The saccus endolymphaticus is variably positive, though its glands stain very deeply (11 +). The walls of the semi-circular canals stain only lightly, but the inner lining is strongly positive.

The rudiments of the Eustachian tube and of the tympanic membrane and cavity at first share the phosphatase content of the tissues from which they form, but by the eighth day they become negative.

The eye.—The eye tissue on the fourth day has generally the darkly stained appearance characteristic of most of the brain at this time, except that the median face of the cup is somewhat lighter than the rest. The nerve tract, which inserts into this less positive region of the retina, is intensely positive (10 +). The fibers of the lens core are negative, but their nuclei take a dark stain, as does the epithelial rim. Up to the eighth day the only change in the lens is that the cytoplasm of the epithelial rim loses its reactivity; the nuclei of both core and rim remain at least slightly positive throughout.

In the retina the diminution of phosphatase spreads toward the outer face of the cup on the fifth and sixth days, so that by the eighth day only the thin lenticular zone, the future iris, is markedly active. The bulk of the retina at this time is lightly positive, but the inner layer of optic fibers shows the same high phosphatase content as in the optic tract itself; and in addition there is a darkly staining layer between the ganglion cells and the base of the layer of Müllerian fibers. This reactive layer appears to include the inner plexiform fibers and also some very large cell bodies which may be the amacrine cells. In the formation of this layer both the histological differentiation and the corresponding phosphatase development spread simultaneously from the fovea outward. The mesenchymatous keel of the pecten is moderately reactive, and the budding eyelids are noticeably darkened.

The nose.—The whole nasal epithelium shares the high phosphatase content of its immediate surroundings on the fourth day, but this gradually diminishes, although the internal limiting membrane continues to stain deeply; the surrounding mesenchyme meanwhile also becomes less reactive (5 +). The condensed mesenchyme from which the nasal processes and turbinals are derived exhibits black deposits (10 +) on the seventh day, just before the cartilage begins to appear, and the nasal septum also shows phosphatase activity before the cartilaginous bar forms. As the cartilage differentiates the phosphatase again disappears.

b. Digestive system

On the fourth day the condensed mesenchyme of the visceral pouch region has a uniformly positive reaction. The lining of the mouth and pharynx stain darkly at this time, but later this epithelium loses some of its phosphatase content so that by the seventh day only the anterior and dorsal regions of the mouth lining are positive. The muscular mass of the tongue reacts moderately at the base at first, but the more anterior portion as well as the surface is only slightly positive. By the eighth day only the epithelium of the front component shows any reaction. The epithelial cords of the thyroid stain heavily throughout (Fig. 16), and the reaction is intrinsic rather than due, as one might suspect from Gomori's (1941a) results on adult mammalian thyroids, to the presence of preformed phosphate in the colloid.

The alimentary tract itself is at first moderately positive throughout, but its positivity declines steadily, with the diminution of phosphatase affecting the more posterior parts of the tract first. The reactivity of the oesophagus wanes after the period of occlusion, although it has a thin lining membrane that continues to stain heavily (Fig. 16). Between the fifth and eighth days a decreasingly positive re-

action extends through the duodenum, the lower parts of the intestine meanwhile becoming completely negative in the cytoplasm. The glands of the proventriculus include some of the positive lining membrane when first seen on the sixth day, but on the following day are quite negative (Fig. 18). On the eighth day the cytoplasm of the entire alimentary epithelium is negative, and even the nuclei, which

TABLE I

Relative phosphatase activities of digestive tract and derivatives

(For explanation of values, see page 53)

The endoderm generally has an alkaline phosphatase rating of 5+ at two to three days, acid phosphatase 1+. Mesoderm: alkaline phosphatase 7+, acid phosphatase 2+.

Tissue	Alkaline			Acid		
	4 days	6 days	8 days	4 days	6 days	8 days
Oesophagus—epith.	7+	5+	3+	1+	2+	2+
mucosa	8+	5+	4+	1+	1+	1+
Crop —epith.		4+	3+		2+	2+
mucosa		5+	3+		1+	2+
Stomach —epith.	5+			2+		
mucosa	8+			3+		
Proventriculus—epi.		4+	3+		3+	3+ (body) 5+ (glands)
muc.		7+	7+		1+	4+
Gizzard—epith.		5+	4+		2+	4+
mucosa		7+	4+		2+	2+
Small intestine—epi.	3+	2+	2+	2+	2+	4+
muc.	8+	7+	4+	2+	1+	1+
Large intestine—epi.	4+	2+	1+	2+	2+	2+
muc.	8+	7+	4+	1+	1+	trace
Rectum	3+	2+	2+	1+	1+	trace
Cloaca	1+	1+	1+	trace	trace	trace
Pancreas	4+	4+	3+*	2+	3+	3+
Liver cells	7+	5+	2+	4+	5+	6+
Liver endothelium	9+	10+	12+	trace	trace	trace
Ductus choledochus		3 to 8+	3 to 6+	2+	3+	4+
Thyroid	10+	12+	12+	3+	5+	7+
Trachea	8+	5+	4+	2+	3+	4+
Bronchi	8+	5+	4+	2+	3+	4+
Mesobronchi	8 to 3+	7 to 3+	2+	2+	3+	4+
Entobronchi		3+	3+		2+	2+
Mesoderm of lung bud	8+	7+	4+	2+	1+	1+

* In tubules, about 8+.

were originally strongly positive, retain only a small part of their former reactivity. The phosphatase deposits also disappear from the linings of the crop and proventriculus, but those of the gizzard and duodenum remain positive. The cloaca is negative throughout.

The closely packed mesenchyme surrounding the developing alimentary tract gives a moderately strong reaction at first, and this continues to be true on the fifth

and sixth days, as the mesenchyme becomes differentiated into a layer of loosely packed tissue (mucosa) immediately around the digestive tube, and a denser layer of circularly arranged cells (muscularis mucosa) outside the first. By the eighth day, however, the muscular layer has lost most of its phosphatase, except for small amounts in the nuclei, but the inner layer continues to be reactive (Fig. 18).

The pancreatic tissue, as it differentiates, becomes less reactive than the mesoderm in which it lies. On the sixth or seventh day, however, phosphatase begins to appear in the organ itself, and on the eighth day, when a glandular structure begins to develop, the tubules are strongly positive. The liver has a strong reaction at four days, but with the progress of differentiation the center of the mass of tissue first becomes negative, and this negativity gradually extends to the periphery; by the eighth day even the nuclei of the hepatic cells have only a trace of activity. During the same period, however, phosphatase develops in the endothelium lining the sinusoids, the reaction appearing first around the principal venous channels and then spreading through all the liver spaces (Fig. 22). The ductus choledochus is reactive within the liver, but becomes negative as soon as it emerges from the liver substance.

The trachea is strongly positive on the fourth day, but the reactivity wanes steadily. The more proximal portions of the bronchi react similarly, but it can be seen on the fifth day that the phosphatase content diminishes sharply at the bend between the anterior and middle mesobronchi, and again between the middle and posterior mesobronchi. Both trachea and mesobronchi have an extremely black inner lining at first, but this too gradually loses its enzyme content. The entobronchi are almost negative throughout.

If the trachea of the hatched chicken has the same high concentration of phosphatase in its ciliary border as Bourne (1943) has described for the trachea of the rat and guinea pig, the accumulation of phosphatase must occur after the eighth day.

c. Urogenital system

Although it differentiates from active mesenchyme, the cytoplasm of the Wolffian body is generally weakly positive on the fourth day. The only parts which show an intense reaction are the necks of the tubules, and the nuclei throughout. The cytoplasm of the duct itself is clear along its entire length. As the structure differentiates further, a high concentration of phosphatase accumulates in the brush borders of the secretory tubules, while the quantity of the enzyme diminishes simultaneously in the outer borders of these tubule cells. The collecting tubules, Bowman's capsules, and the glomeruli, as well as the Wolffian duct, have little or no phosphatase during this period. The Müllerian duct however stains quite darkly (Fig. 20). The metanephric blastema is moderately positive, but the tubules that differentiate from it are only weakly so; the blastema of the ureter, on the other hand, contains only a little phosphatase, and the tube itself shows no change in this respect.

The gonads are quite reactive when they first appear, but their phosphatase content diminishes steadily. The same is true of the suprarenals. Neither organ had assumed a pattern of phosphatase distribution, as the findings of Gomori (1941a) and Kabat and Furth (1941) suggest that they might at a later stage.

TABLE II

Relative phosphatase activities of the urogenital system

At 2 to 3 days: mesoderm from which Wolffian body is formed has alkaline phosphatase rating of 8+; acid phosphatase, 2+.

Tissue	Alkaline			Acid		
	4 days	6 days	8 days	4 days	6 days	8 days
Glomeruli	3+	3+	3+	1+	1+	1+
Bowman's capsules	5+	3+	3+	trace	trace	trace
Secretory tubules—walls	10+	6+	3+	4+	4+	4+
—brush border	10+	14+	14+	4+	4+	4+
Collecting tubules	4+	trace	trace	2+	2; 8+	2; 8+*
Wolffian duct	4+	3+	2+	8+	8+	8+
Müllerian duct		8+	5+		2+	2+
Metanephric blastema		8+	8+	2+	2+	2+
Metanephric tubules		4+	4+	8+	8+	8+
Ureter	4+	4+	3+	2+	2+	2+
Suprarenal (provisional cortex)	8+	6+	4+	1+	1+	2+
Gonad	10+	8+	6+	2+	3+	4+

* The phosphatase content of the tubule rises sharply at its entrance into the Wolffian duct.

d. Circulatory system

The heart.—The cardiac muscle continues to be completely free of alkaline phosphatase; even the nuclei, which stain lightly on the fourth day, become completely negative by the eighth day, except that the nucleoli are still reactive. Thus a clear-cut boundary appears in the bulbus between the negative heart tissue and the phosphatase-containing tissue external to it. The thin endothelial linings, which by the fifth day reach into the deepest recesses of the trabeculae, stain solid black (Fig. 23). High concentrations of phosphatase are also found in the septa, including the cushion septum, a development which is already foreshadowed on the third day by thickening of the intensely reactive epithelium around the valves. The thick spongy lining of the bulbus shares this positive reaction (Fig. 5); in fact, all the vessels entering or leaving the heart are lined with phosphatase containing tissue.

The blood vessels.—When first laid down, all the blood vessels share the high reactivity of the condensed mesenchyme that forms them (Fig. 7). As late as the eighth day the still poorly differentiated small arteries are reactive throughout, but the walls of the aorta and the larger arteries gradually become free of phosphatase as the muscular tissue differentiates. However the pulmonary arteries, and the aorta and its major branches (Fig. 18), are lined with highly reactive endothelium. The umbilical artery has virtually no phosphatase by the eighth day, while the omphalomesenteric arteries have a reactive endothelium where they branch from the aorta, but this becomes negative as they pass outside the body.

The veins are not generally reactive (Fig. 16). The ducts of Cuvier and the sinus venosus, however, and later the proximal portions of the venae cavae, possess

the same phosphatase-rich lining as the heart itself. Likewise the meatus venosus and hepatic veins are lined with the heavily staining tissue that runs through the sinusoids, and the omphalomesenteric veins remain moderately positive throughout the period studied. The larger umbilical vein is rather strongly positive. Except for the capillary bed of the liver, the walls of the capillaries are by the eighth day not more reactive than the tissues that form them.

The presence of alkaline phosphatase in vascular endothelia at the early stages at which it has been observed is of interest in relation to the question of the origin of endothelial phosphatase. It is sometimes held that endothelial cells pick up phosphatase from the serum. If Armstrong and Banting (1935) are correct, however, in their contention that bone is the source of serum phosphatase, it is clear that phosphatase in the endothelium of six- or seven-day embryos cannot be derived from serum; more likely the endothelia, like other tissues, produce their own phosphatase.

The spleen has a weak, uniform reaction through the eighth day, at which time it is still in an early state of differentiation.

e. The skeleton

The hind limbs from the sixth to the tenth day were studied in most detail; pairs of limbs were fixed on each day, plus pairs of feet for the eleventh day, one member of each pair being used for phosphatase demonstration and the other for the demonstration of phosphate deposited *in situ*. All specimens were sectioned transversely.

The limb buds on the fourth day are merely masses of condensed mesoderm which share the high phosphatase content common to mesoderm at this time (8 to 10+). On the fifth day each mass becomes differentiated into an inner portion, the rudiment of the cartilage, which is more condensed and highly reactive; and an outer portion, destined to form muscle and connective tissue, which is comparatively rarified and less reactive. As the precartilages become transformed into protochondrium, their phosphatase content diminishes sharply except at the periphery, where a thin layer of compressed cells remains positive (8+); within the mass the nuclei continue to show a fairly strong reaction, but the cytoplasm and developing matrix become quite negative. During the protochondrial stage the enveloping layer of phosphatase-containing cells increases in thickness without giving a more positive reaction; but with the appearance of true hypertrophic cartilage the perichondrium begins to develop the most intense reaction found in the early embryo (16+); the cells affected are the perichondrial osteoblasts.

As is true of histological differentiation, the accumulation of phosphatase begins, in the long bones, midway along the diaphysis, and spreads gradually toward the epiphyses; simultaneously the layer increases in thickness, becomes open and spongy (Figs. 12 and 14), and its inner edge invades the matrix raggedly. In the fibula phosphatase appears throughout the diameter of the shaft toward the distal end, but where typical epiphyses occur the phosphatase accumulation stops abruptly at the zone of flattened cells (Fig. 11). When the phosphatase-laden osteoid has achieved a thickness in the diaphysis roughly equal to half the radius of the enclosed cartilage, thin shells of bone are laid down near the inner surface of the layer (Figs. 13 and 15). From Table III, which summarizes the development of carti-

lage, phosphatase, and bone in the hind limb from the sixth to the eleventh day, it will be seen that bone deposition follows inevitably when a certain level of cartilage differentiation is reached; this is equally true for the tibia, in which the development of the cartilage and osteoid envelope is rapid, and for the proximal phalanges, in which hypertrophy and phosphatase accumulation are slow.

It was pointed out above that the first differentiation of cartilage from mesoderm is accompanied by a marked diminution of the primitive embryonic phosphatase in

TABLE III

Summary of cartilage, alkaline phosphatase, and bone formation

Day	Structure*	Condition of cartilage	Condition of perichondral phosphatase layer	Deposition of perichondral bone
6	tibia, fibula	precartilage	light (8+)	−
	femur	precartilage	light (8+)	−
	metatarsals	mesenchyme	—	−
7	phalanges	precartilage	light (8+)	−
	metatarsal 1	mesenchyme	—	−
	metatarsal 2–4	protochondrium	2-thin, compact (12+)	−
			3, 4-thick, compact (16+)	−
	tibia, fibula	hypertrophic	thick, compact (16+)	−
	femur	hypertrophic	thick, compact (16+)	−
8	phalanx 1	precartilage	light (8+)	−
	phalanx 2–4	protochondrium	thin, compact (12+)	−
	metatarsal 1	precartilage	light (8+)	−
	metatarsal 2–4	hypertrophic	thick, spongy (16+)	3, 4+; 2−
	tibia, fibula	hypertrophic	thick, spongy (16+)	+
	femur	hypertrophic	thick, spongy (16+)	+
9	phalanges	protochondrium	thin, compact (12+)	−
	metatarsal 1	protochondrium	thin, compact (12+)	−
	metatarsal 2–4	hypertrophic	thick, spongy (16+)	+
	tibia, fibula	hypertrophic	thick, spongy (16+)	+
	femur	hypertrophic	thick, spongy (16+)	+
10	phalanges	hypertrophic	thick, spongy (16+)	−
	metatarsal 1	protochondrium	thin, compact (12+)	−
11	phalanges	hypertrophic	thick, spongy (16+)	+

* The most advanced portion of the structure is considered.

the cytoplasm. Shortly afterward the nuclei in the zone where hypertrophy occurs also undergo loss of phosphatase, so that the cells of the cartilage beginning to hypertrophy are negative. Already on the seventh day in the tibia and femur, however, some of these cells develop a very intense reaction while other neighboring cells continue to be negative; at the same time the matrix begins to exhibit signs of phosphatase activity. The reactive cells seem to decrease in number through the tenth day, and they are most numerous midway along the diaphysis. Possibly these cells are engaged in synthesizing the phosphatase that becomes con-

centrated at the periphery, or they may be endochondral osteoblasts, for erosion has begun in advanced cartilages on the tenth day (Fig. 14).

The anlagen of the vertebral bodies and neural arches pass through the same stages of cartilage differentiation as do those of the long bones, at about the same time, although by the eighth day the hyaline cartilage of the axial skeleton has achieved an advanced state of differentiation only immediately around the notochord (Fig. 7). Each separate element is enveloped by a compressed layer of elongate cells which are more reactive than those within the cartilage; this layer resembles that which envelops the small-celled cartilage of the long bones, but it never has anything like the tremendous phosphatase activity of the perichondrium of ossifying long bones. The cell bodies are generally only slightly reactive, but in the anterior region on the eighth day a number of reactive cells appear among the negative ones in the center of each future vertebra, where endochondral ossification will begin several days later. In a few sections through the neck of an eleven-day embryo it was observed that both matrix and cells of the cartilage close to the notochord contained a very high concentration of phosphatase, while the remainder of the vertebral cartilage contained none of the enzyme; ossification had not begun.

No detailed study was made of the development of the girdles, but it was noted that on the eighth day the coraco-scapula is covered by a phosphatase-rich perichondrium (14 +) similar to that of the limb bones; the layer is thicker on the coracoid than on the scapular part. The clavicle at the same time is a mass of mesoderm heavily impregnated with phosphatase. The elements of the pelvic girdle show practically no perichondral phosphatase accumulation at eight days. The ribs however are heavily coated with strongly reactive phosphatase in the diaphyseal regions (Fig. 20).

The development of the skull was not studied.

f. Other tissues

Notochord.—The nuclei of the notochord are less intensely reactive on the fourth day than at earlier stages, and the cytoplasm is negative. Within the next two days the nuclei also lose the remainder of their phosphatase content.

Muscles.—As the fibers of the skeletal muscle differentiate, their cytoplasm loses entirely the phosphatase content of the primitive mesoderm from which they are formed. The nuclei lose most of their phosphatase, but even on the tenth day they are still slightly reactive.

Skin.—The superficial ectoderm is intensely reactive at the beginning of embryogenesis, but already by the fourth day the phosphatase content of the cytoplasm has diminished sharply, and by the seventh day even the nuclei are negative. At the same time the thick mesodermal corium has also become negative. This change is not uniform, but begins first in the dorsal and anterior regions of the body.

Mesenchyme.—The loose mesenchyme distributed throughout the embryo is positive (8 +) as late as the fifth day, but thereafter it gradually loses its enzyme content. On the eighth day this tissue is almost negative in the body, but in the head masses of it which are presumably concerned in the ossification of membrane bone remain strongly positive.

Feather germs.—Undifferentiated feather germs were found on ten-day hind limbs. They contained a core of positive mesoderm (8 +).

II. Acid Phosphatase

Acid phosphatase is widespread in the early embryo, but in virtually all cases in apparently far lower concentration than alkaline phosphatase. With Gomori's (1941b) unmodified technique only the Wolffian duct and some of the kidney tubules ever produced a black deposit; in all other organs the deposits, even after 16 hours' incubation, took merely a golden-brown stain of varying intensity. As has been noted before, the extent and intensity of staining varied considerably among different specimens of the same age, and in spite of the preparation of a large number of specimens it was not easy to decide how much of the variability is intrinsic and how much due to the troublesome lability of the enzyme. The use of ascorbic acid as an activator improved the uniformity of the results but, as will be explained later, it did not entirely correct the inconstancies. Several embryos even failed to produce any deposits at all, for no readily apparent reason. When deposits did appear, however, their distribution and relative concentrations in the separate organs and throughout the body as a whole were quite constant.

Acid phosphatase is commonly found in nuclei, but unlike the alkaline enzyme it occurs only in uniform distribution in the karyoplasm, which takes a diffuse stain; generally in reactive cells, however, there is a darkly stained cap, which may be the Golgi apparatus, closely applied to the surface of the nucleus (cf. Moog, 1943). Bourne (1943) found such caps stained for alkaline phosphatase in the nuclei of certain intestinal cells; they were not observed in the alkaline-stained cells of the embryo, but the heavy reactions of the nuclear sap and membranes may have obscured them. On the basis of the relative distribution of the enzyme in nucleus and cytoplasm the same three classes of tissues may be defined as were described for alkaline phosphatase; but in addition, in the differentiation of some tissues acid phosphatase disappears almost completely from the nuclei as well as from the cytoplasm.

The difference in the nuclear staining is one argument against the possibility that the acid enzyme of this study is merely residual activity at acid pH of the much stronger alkaline enzyme. And this possibility is completely eliminated by several other facts. First, the alkaline enzyme is entirely suppressed at pH 8. Second, at later stages the two enzymes assume distributions that differ in many important respects. Third, at early stages as well as later, the acid enzyme is inhibited by fluoride, which has been shown to have no effect on the alkaline enzyme. And last, as will be demonstrated in a subsequent section, the two enzymes are affected in opposite fashions by a variety of chemical substances.

The first day

In the unincubated blastoderm the smaller yolk granules, whether in the superficial layers of the yolk or enclosed within the cells, show a moderately high phosphatase content, while the deeper layers of yolk are less reactive; but as in the case of the alkaline phosphatase, proximity to the blastoderm rather than the nature of the yolk, seems to determine the enzyme content. The central area of the ectoderm is the most reactive part at this time (about 3 +); the reactivity of the cytoplasm wanes somewhat near the germ wall. The endoderm has little cytoplasmic phosphatase, although the yolk granules enclosed in the cells cause it to stain no-

ticeably. The nuclei are difficult to detect, and do not seem to be stained more deeply than the cytoplasm.

As the primitive streak differentiates, the ectoderm composing it becomes more reactive (4 +), and the demarcation between the central ectoderm and the less reactive periphery becomes sharper. The endoderm stains more intensely than in the gastrula stage, and the mesoderm is intermediate in enzyme content between the ectoderm and endoderm; as the yolk granules disappear it can be seen that in the mesoderm and endoderm the nuclei are more reactive than the cytoplasm, but in the ectoderm the intensity of the cytoplasmic stain obscures the condition of the nuclei (Fig. 1). The head process ectoderm and the notochord share the high phosphatase content of the primitive streak ectoderm as soon as they are laid down, while the surrounding extra-embryonic tissue continues to be only weakly reactive (1 +). The activity of the lateral mesoderm increases when the somites begin to form.

Two and three days

The nervous system continues to be the most reactive on the second day; the notochord is also quite reactive, but the other tissues of the body are markedly weaker (2 +), although their nuclei stain rather deeply. The extra-embryonic tissues become negative at a short but variable distance from their confluence with the body. The same antero-posterior gradient obtains as was noted in the case of alkaline phosphatase.

On the third day the spinal cord is as rich in phosphatase as before, and in the ependyma sometimes even more so (4 +). In certain regions of the brain, however, there is a sharp decrease. The hindbrain continues to be reactive, especially where it passes into the spinal cord, but the midbrain is largely unreactive, except in its ventral region, and the diencephalon too remains positive only on its roof and around the point where the optic stalks enter. Of the telencephalic vesicles only the anterior faces react. The nasal pits, optic cups and lenses, and otocysts continue to be positive, though less so than the spinal cord.

The loose mesenchyme is negative, but condensed aggregations of mesodermal cells remain lightly positive (2 +). There is a sharp increase concurrent with the appearance of the Wolffian body, however, and already at the end of the third day the Wolffian duct is quite rich in acid phosphatase (8 +). The endoderm continues to be weak except at the points of the liver and allantoic diverticula: the liver tissue is extremely reactive (4 to 5 +) when it first appears, and the allantois is equally reactive; the mesoderm of the allantoic bud shares the strong reaction of the endoderm.

Four to eight days

a. Nervous system

1. *Brain and cranial ganglia:*

The brain continues to show some acid phosphatase activity through the fifth day. At that time the mid- and hindbrain are about equally reactive (2 +), with the ependymal layer being more positive than the rest; otherwise there are no clear-cut local differences. The bulk of the diencephalon contains only a trace of acid phosphatase, but the dorso-lateral walls, and the ventral portion where the optic

nerves enter, are slightly more reactive (1 +). The cerebral hemispheres can also be rated at 1 + to 2 +, except that the partition which separates them is negative. On the sixth day the quantity of acid phosphatase in the brain is still slighter, and on the seventh and eighth days only with the aid of ascorbic acid could traces of the enzyme be detected. Similarly the acid phosphatase content of the cranial ganglia falls to a mere trace by the eighth day.

2. Spinal cord:

The general phosphatase activity described for the spinal cord (Moog, 1943a) is of the order of 2 + to 3 +, with the concentrations in the motor horns and ependyma being considerably stronger (4 + to 5 +). The activity of the large cells of the ganglia can also be rated at 5 +.

3. Sense organs:

The sense organs in their earliest expressions share the phosphatase content of the ectoderm from which they are formed. Their reactivity wanes rapidly, however, and by the sixth day they are all devoid of phosphatase, except that traces remain in the retina.

b. Digestive system

The condensed mesenchyme of the visceral pouch region exhibits only slight activity on the fourth day, and even this small activity wanes subsequently. The epithelial lining of the mouth is almost negative, but the inner surface of the pharynx and the laryngotracheal groove retain some acid phosphatase activity throughout this period. The thyroid stains heavily, according to the same pattern as with alkaline phosphatase.

The oesophagus gains somewhat in activity on the fifth or sixth day (Fig. 6), attaining a moderate phosphatase content which it shares with the crop; the inner lining membrane does not stain more deeply than the cells. The mesenchyme surrounding the oesophagus is uniformly weak, but that around the crop by the eighth day exhibits a relatively high phosphatase content in the innermost layer of compact tissue; the intermediate layer of loose mesenchyme and the outer layer of involuntary muscle fibers are much less active. The stomach and intestinal endoderm increase markedly in enzyme content up to the eighth day. The body of the proventriculus accumulates considerable phosphatase, and the glands become even richer (Fig. 19); the outer mesenchyme shows the same reactions as just described. The endoderm of the gizzard is also increasingly active, but its mesenchyme, which is less differentiated than that further anterior, is only slightly active on the eighth day. The small intestine shows the same relations as the gizzard, but the endoderm of the large intestine and rectum continue to be only weakly positive, and their accompanying mesenchyme contains only traces of phosphatase. The cloaca develops a very small enzyme content during this period.

The liver cells continue to gain in phosphatase content as the organ grows (Fig. 19), the increase proceeding from around the large veins to the periphery; the sinusoidal linings however do not contain the acid enzyme. The ductus choledochus is positive, but less so than the liver tissue surrounding it. The pancreas is at first

only slightly positive, but as in the case of alkaline phosphatase there is some accumulation of the acid enzyme as the glandular structure begins to differentiate.

The trachea and the respiratory tubes which spring from it undergo the same small increase in phosphatase content as occurs in the digestive tract generally between the fourth and the eighth day (Fig. 6), though the entobronchi remain less positive than the rest. The mesoderm of the lung bud, on the other hand, is only faintly reactive throughout.

c. Urogenital system

The Wolffian duct continues to be the most phosphatase-rich organ in the body (Fig. 6). The tubules are at first equally reactive throughout, but by the fourth or fifth day the secretory portions of the tubules begin to show a heightened reaction both in the brush borders and throughout the cells of the tubule walls; this is the same condition as Gomori (1941b) found in the kidneys of several adult mammals. The collecting tubules are definitely less reactive except at their entrance into the Wolffian duct, where they suddenly exhibit the intensity of the duct itself. The glomeruli are slightly positive, and Bowman's capsules have merely a trace of activity. The Müllerian duct is moderately positive (Fig. 21). The metanephric blastema is only lightly positive, but the tubules display very high activity, almost equal to that of the Wolffian duct, as soon as they appear. The ureter is weakly positive.

The gonads seem to possess a uniform overall phosphatase content during the period under consideration. But by the eighth day the enzyme is concentrated principally in the cortex and sex cords, the interstitial substance being much less reactive (Fig. 21). The suprarenal increases slightly in reactivity by the eighth day, but, as is also true of the alkaline enzyme, close examination fails to reveal differentially stained elements which might be the chromaffin cells.

d. Circulatory system

With the exception of the spongy lining tissue of the bulbus, which regularly demonstrates a small phosphatase content, all parts of the circulatory system have generally been found to be negative (Fig. 6). In one case, however, in which freshly prepared specimens were incubated for 18 hours in the presence of 0.01 M ascorbic acid, traces of acid phosphatase were found in all parts of the circulatory system; there appeared to be no noteworthy local differences. The red blood corpuscles are negative.

The spleen has a low phosphatase content throughout the eighth day.

e. Other tissues

The loose mesenchyme has only a trace of acid phosphatase activity, but the condensed masses of the tissue retain their phosphatase content until differentiation occurs. The membranous anlagen of the cartilages are thus somewhat positive (2 +) on the sixth day, but as the definitive cartilage appears it is negative, except for small amounts of phosphatase in the cell nuclei. The acid enzyme does not appear to have any further role in the differentiation of cartilage bones; its possible relation to the differentiation of membrane bones was not examined.

The striped muscles similarly lose practically all of their acid phosphatase content as they differentiate from reactive mesoderm.

The notochord is virtually negative by the fifth day, except for traces of phosphatase remaining in the nuclei.

The skin also loses most of its primitive reactivity by the sixth day, after which time all layers are negative, except, again, for slight reactivity in the nuclei.

Chemical Tests

The purpose of determining the effects of various chemical reagents on the activity of the embryonic phosphatases is threefold: first, to discover to what extent the embryonic phosphatases are identical with the phosphatases which have already been found in a variety of tissues and secretions; second, to find out whether each enzyme is a single entity or shows local variations; and third, to elucidate the differences between the two enzymes, and incidentally examine the possibility that any part of the weak activity of the acid enzyme is merely residual activity at acid pH of the stronger alkaline enzyme. Six-day embryos are most suitable for these purposes, since at this stage both alkaline and acid enzymes have to a certain degree assumed what appears to be their definitive distribution in partly differentiated organs, yet at the same time large amounts of both enzymes are still present in their primitive state in undifferentiated tissues. In order to secure a large number of comparable slides for treatment, a ribbon 8 micra thick was cut from the embryo and consecutive sections in groups of two or three placed on slides; in this way a series of 12 or more slides all showing practically the same tissues could be obtained. Such series were made at the eye, fore-limb, mid-body, and hind-limb level. Incubation was continued for 1½ hours in tests for the alkaline enzyme, 15 hours for the acid enzyme.

pH.—The alkaline phosphatase activity was weakened at pH 8.6 and abolished at pH 8.0. No acid phosphatase activity was obtained at pH 4.7 or pH 5.4.

Mg^{++}.—0.01 M $MgCl_2$ markedly activates the alkaline enzyme, at least doubling its production of phosphate during a one-hour run. A 0.001 M solution has a slighter effect. A 0.01 M solution does not affect the activity of acid phosphatase. These results are in agreement with the generally accepted hypothesis that Mg^{++} is a coenzyme of the alkaline phosphatase, but has no relation to the acid enzyme.

Mn^{++}.—The peculiar effect of $MnSO_4$ on the precipitate formed under the influence of acid phosphatase has already been reported (Moog, 1943b); it was concluded that no true activation is involved. The effect of $MnSO_4$ on the alkaline enzyme could not be tested, since its addition to the alkaline incubation solution causes the formation of a precipitate.

Zn^{++}.—Hove, Elvehjem and Hart (1940) reported that Zn^{++} increases the activity of crude alkaline intestinal phosphatase, decreases that of kidney and bone, and inhibits all three after dialysis. In the embryo 0.001 M $ZnSO_4$ greatly inhibits the activity of alkaline phosphatase, while 0.01 M abolishes the activity. Neither concentration had any effect on acid phosphatase.

F^-.—NaF is commonly accepted as an inhibitor of acid but not of alkaline phosphatase. 0.01 M NaF completely inhibited the acid enzyme in these studies; it could not be tested against the alkaline enzyme because of the very low solubility of CaF_2.

Sodium glycocholate.—Bodansky (1937) reported that various bile acids, including glycocholate, inhibit bone and kidney, but not intestinal, alkaline phosphatase; also Schmidt and Thannhauser (1943) found intestinal phosphatase to be unaffected by bile acids. In nine-day embryos 0.01 M Na glycocholate completely suppressed the activity of alkaline phosphatase in heart and liver endothelia, nerve and osteoid tissue, kidney and gonad, and also in the epithelium, brush border, and mucosal mesenchyme of the small intestine.

Phloridzin.—Lundsgaard (1933) originally showed that phloridzin inhibits both phosphorylation and dephosphorylation by kidney phosphatase. Later Kalckar (1936) and Beck (1942) substantiated the claim that phloridzin suppresses the phosphorylation of glucose by kidney extracts at neutral pH; but Beck also reported that phloridzin inhibits the hydrolytic activity of kidney phosphatase at pH 5 but has no effect at pH 7–9; the latter finding is in agreement with the results of numerous other workers, including Kritzler and Gutman (1941), who found that 0.01 M phloridzin has no effect on the alkaline phosphatase activity of the rat kidney in either the direct chemical or histochemical technique. The embryonic alkaline phosphatase, however, was very strongly (though not quite completely) inhibited by 0.01 M phloridzin, whereas the acid phosphatase was but slightly affected by the same concentration.

Reducing substances.—Albers (1935) presented extensive evidence to show that kidney phosphatase is inhibited by sulfhydryl compounds, and his results have several times been substantiated wholly or in part (del Regno, 1939; Pyle, Fisher and Clark, 1937; Schmidt and Thannhauser, 1943). In this study it was found that the alkaline phosphatase activity is suppressed completely by 0.01 M glutathione and by 0.01 M cysteine hydrochloride; and it is also inhibited to a marked extent (estimated as 50 per cent) by 0.01 M ascorbic acid. (Possibly rapid inactivation of ascorbic acid at pH 9.3 accounts for the incompleteness of this inhibition; however, Kiese and Hastings (1938) found that in runs lasting less than an hour 0.01 M ascorbic acid also had only a slight effect.) Acid phosphatase could not be tested with the two sulfhydryl reagents, since their addition to the acid incubating solution caused the appearance of a heavy precipitate. But 0.01 M ascorbic acid activated the acid phosphatase in freshly prepared specimens to an extent that seemed reasonable to estimate at 300 per cent.

From the effect of ascorbic acid it was first thought that the loss of acid phosphatase activity that histochemical preparations commonly undergo might be due entirely to oxidation. Therefore the effect of ascorbic acid on sections which had been standing at room temperature for one week was compared with that on freshly prepared sections. Activation occurred in the older material, but the level of activity was not as high as that of activated fresh preparations. Hence it appears that the spontaneous loss of acid phosphatase activity is not due principally to oxidation reversible by ascorbic acid.

Oxidizing agents.—The strong effect of ascorbic acid on acid phosphatase nevertheless indicated the advisability of determining the effect of oxidizing agents on the enzyme. Although Barron and Singer (1943) have reported that acid phosphatase is not inhibited by sulfhydryl oxidants, the activity of the enzyme in the presence of 0.01 M iodoacetic acid was tested: there was no clearcut inhibition. On the other hand, the activity of alkaline phosphatase was almost completely suppressed by 0.01 M iodoacetic acid.

It was then decided to try the effect of a less specific oxidizing agent. In the case of the alkaline enzyme, 0.01 M potassium ferricyanide exerted a limited but definite inhibition, estimated as 30 to 40 per cent. In the case of the acid enzyme, complete inhibition occurred. This inhibition was quite reversible, as was shown by exposing sections to incubating solution containing ferricyanide for two hours, and then transferring to plain incubating solution; the deposits produced were equal to those produced in control sections not exposed to the oxidizer. According to Sizer, both acid and alkaline phosphatase are inactivated by only such oxidizing agents as have E_h values between + 400 mv. and + 600 mv., the range in which 0.01 M ferricyanide probably falls; the fact that the E_h would be higher in acid than in alkaline solution may thus account for the more severe effect on the alkaline enzyme.

Taken together, these results with oxidizers and reducers offer little support for the view that sulfhydryl groups are important in the functioning of phosphatases. Particularly in the case of the alkaline phosphatase, that view does not seem reconcilable with inhibition by both iodoacetic acid and —SH compounds. The acid phosphatase moreover has now at least twice been shown to be insensitive to the effects of sulfhydryl oxidants.

The purposes of the chemical tests have been clearly fulfilled. In the first place, it has been shown that the two embryonic enzymes correspond well with the patterns that extensive research has delineated for alkaline and acid phosphatases generally. With one important exception, such differences as have been indicated can be explained as being due to differences in the state of purification of the enzymes; the exception is of course the action of phloridzin. Presence or absence of protective substances, coactivators, etc., probably cannot be called into account here, at least for the alkaline enzyme, for Kritzler and Gutman made histochemical tests of the latter on material prepared just as in this study, except that they used alcohol as fixative. The situation is the more difficult to understand because the kidney phosphatase has by the sixth day assumed what has been repeatedly shown to be its definitive distribution (Gomori, 1941a; Kabat and Furth, 1941; Krugelis, 1942), and furthermore may be assumed to be functional; yet the embryonic kidney phosphatases do not give the same response to phloridzin as other kidney phosphatases have been found to give.

Secondly, it has been amply demonstrated that the two embryonic phosphatases are separate substances. In addition to the fluoride sensitivity of the acid enzyme, it has been shown that the two enzymes differ, both in primitive and in differentiated tissues, in their responses to Mg, Zn, phloridzin, ascorbic acid, and iodoacetic acid.

Thirdly, no evidence has been found to indicate that each enzyme is other than a single entity. Taking into consideration the varying concentrations of the enzymes in different tissues, it may be stated that in no case was one tissue affected more or less than others by any reagent. This finding is in agreement with most current work, for no attempt to fractionate the phosphomonoesterases which Folley and Kay (1936) have classified as AI (alkaline) and AII (acid) has succeeded, except that of Bodansky, cited above, whose results may be due to factors extrinsic to the enzyme proper.

Lastly, no differences have been found between nuclei and cytoplasm in regard to the characteristics of either phosphatase. This result was partly to be expected from the fact that the nucleotidase which Levene and Dillon (1930) and others have found in numerous animal tissues is now accepted as being identical with the common alkaline phosphomonoesterase, with which Folley and Kay indeed classified it. The acid phosphatase of nuclei, on the other hand, has apparently been neglected; the evidence presented here certainly indicates that it does not differ from the cytoplasmic acid phosphatase.

Discussion

The most significant result to emerge from this study may be the fact that all primitive embryonic tissues, and especially all nuclei at early stages, contain phosphatase, both alkaline and acid. That phosphatase may occur in tissues which are far from assuming their definitive function has been demonstrated before by Kabat and Furth (1941), who found the substance in the mesenchymal anlagen of the leg and tail in the mouse embryo; the present results suggest that phosphatase may be an invariable concomitant of primitive tissue. It seems not unlikely that the function of this phosphatase may in part at least be concerned in cell division. In the chick embryo both alkaline and acid phosphatase are active in almost every tissue during the stage of intense cell proliferation; but there are exceptions. The extra-embryonic cell layers are very poor in both enzymes at the stage when they are expanding rapidly over the yolk, and the myocardium is all but devoid of phosphatase during a long period of rapid nuclear proliferation. Similarly, other workers have found that active tumors do not necessarily contain much phosphatase (Gomori, 1941b; Kabat and Furth, 1941). The traces of the enzymes that persist in the heart nuclei may of course be sufficient for the mitotic needs of the tissue. Willmer (1942) has indeed shown that in cells of chick heart tissue grown *in vitro,* alkaline phosphatase activity is intensified during mitotic activity and then regresses, leaving the cell negative except for reaction in the nucleolus and centrosphere. But if the very small amount of phosphatase demonstrated by Willmer, or observed in the sectioned heart muscle, is the actual requirement of mitotic activity, then a question remains: what is the function of the excess of phosphatase which the embryo possesses above this basic minimum? In the central nervous system, for example, phosphatase is not limited to the ependyma, where proliferation occurs; and even the undifferentiated endoderm, which is relatively weak in alkaline phosphatase, has a modest amount of the enzyme in its cytoplasm, and considerably more in the nuclei.

But proliferation is only one aspect of the function of primordial cells; equally important is the preparation for the histogenetic expression of differentiation. Experimental embryology has shown repeatedly that differentiation proceeds during a long period before its effects are fully realized, for there are a great number of cases in which transplants made at intervals from regions of apparent undifferentiation display increasing ability to manifest their normal prospective value; this is of course the phenomenon which Huxley and de Beer (1934) have termed "progressive chemo-differentiation." What then of the fact that all tissues of the early chick embryo contain substantial amounts of phosphatase during their period of chemo-differentiation leading to the assumption of definite form? It may not be without

significance that phosphatase is abundant in the primitive streak and its associated structures, and appears in the true embryonic structures as soon as they are laid down. Thereafter no tissue becomes nearly free of phosphatase until its definitive form is clearly indicated; this is equally true of cardiac muscle, which is almost free of the enzyme at the time it starts to beat; of the alimentary tract, parts of which become quite free of phosphatase as they become morphologically demarcated; of parts of the brain, of cartilage, etc. Moreover the increase of phosphatase which the hinder parts of the body undergo through the second day, when chemo-differentiation is proceeding actively, is suggestive in this regard, and so is the heightened phosphatase content of the liver diverticula between their appearance and the development of true liver tissue. The relatively high concentrations of acid phosphatase in the nervous ependyma, and the concurrent proximo-distal courses of histological differentiation and phosphatase decline in the developing retina, also seem pertinent. It is interesting, too, that Krugelis (1942), working with mouse testes, observed that the high concentration of alkaline phosphatase in the spermatogonia and spermatocytes declines to negativity as differentiation proceeds. For the time being, one can merely point out these suggestive parallels; but studies now contemplated will examine in more detail the possible interdependences between the progress of developmental patterns and the changes of phosphatase content.

The basic chemical role of phosphatase in development is even more obscure. Yet considering the tremendous importance which current research is attaching to phosphate transfer, it may not be extravagant to suggest that, among the diverse chemical mechanisms that lead to diverse histologies, there is during a considerable portion of primary differentiation an invariable factor involving phosphorylation and dephosphorylation, and so necessitating the presence of phosphatases. Perhaps the synthesis of proteins under the influence of phosphate-bearing nucleic acids involves phosphatase activity; it will be remembered that Caspersson and Thorell (1941) found especially high concentrations of nucleic acid in the cytoplasm of chick embryo cells in the earliest stages of development. This view of course would relate phosphatase activity to form change, rather than to histogenetic differentiation. In any case it will be interesting to learn why two phosphatases are simultaneously present in primitive tissues, and why their relative activities in different regions vary to some extent.

Although the chemical tests have shown no differences between phosphatases in differentiated tissues and in still undifferentiated anlagen, the phosphatases which accumulate in differentiated structures should probably be regarded as existing in a different phase from the primitive enzymes. The former, that is to say, are not merely remnants preserved from the early period, but are in themselves consequent on differentiation. A comparison of activities will illustrate this point. In the case of the alkaline phosphatase, the perichondrium, epithelia of heart and liver, brush borders of secretory tubules, and several other tissues show a level of activity higher than ever attained by the "unspecialized" enzyme of pre-differentiated tissues, and the same is true, in the case of acid phosphatase, of the Wolffian duct, liver cords, metanephric tubules, and so on. In all these tissues histological differentiation seems to entail somewhere in its course a chemical differentiation in the sense of production of phosphatase to be used in the incipient functioning of the organ. Thus the mesonephros, which is functioning already on the fifth day (Boyden,

1924), assumes by that time a phosphatase distribution virtually identical with that which has been described, especially in the case of alkaline phosphatase, for the adult mammalian kidney (Gomori, 1941a, b; Kabat and Furth, 1941). But the alimentary tract, non-functional in the embryonic period, loses much of its primitive epithelial alkaline phosphatase after becoming well-launched on its course of definitive differentiation, and it does not accumulate more up to the eighth day, even though in adult chickens, as well as mammals, parts of the digestive mucosa are extremely rich in the enzyme (cf. Folley and Kay, 1936; Gomori, 1941a; Kabat and Furth, 1941). And again, in organs which do not have one or both enzymes in their adult condition (e.g., the myocardium, or the liver cells in respect to alkaline phosphatase) there is usually a swift disappearance of the enzyme after the first stages of differentiation are completed; though of course it is not contradictory that a few rather well-differentiated organs (e.g., the brain, which is poor in phosphatase in its adult condition) contain phosphatase which has no obvious functional role; in such cases the enzyme may play a part in the more advanced stages of differentiation. Thus the specific organ phosphatases accumulating as differentiation proceeds are not necessarily conterminous in origin with the primitive phosphatases of the first days, and it will probably be profitable to consider them as separate entities.

This concept of a diphasic phosphatase occurrence in young embryos agrees well with the results of Lipmann (1936), who found a peak of alkaline phosphatase content in extracts of whole chick embryos on the sixth day. The histochemical preparations certainly give the impression that there is such a peak between the fifth and seventh days, for at that time the specific organ phosphatases are accumulating rapidly while the phosphatase in undifferentiated tissues, especially mesoderm, is still present in great quantities, although it disappears very soon thereafter with the appearance of cartilage and skeletal muscle.

Among the separate observations reported, those bearing on the relation of phosphatase to ossification deserve to be commented on. Robison's hypothesis that the activity of alkaline phosphatase is a causative factor in the deposition of bone salts is now generally accepted, and my results on the long bones are in full agreement with it. These results demonstrate, as Fell and Robison (1929) have already shown for chick femora cultivated *in vitro,* that the extremely high phosphatase concentration which is associated with ossification begins to accumulate with the differentiation of hypertrophic cartilage, and that the phosphatase concentration from seven to ten days is largely confined to the perichondrium, where it is limited to the diaphysial region between the zones of flattened cells—the exact region in which bone is seen to be deposited. Further, it is clear that in all bones of the hind limb the deposition of calcium phosphate is preceded by the development of a broad zone of high phosphatase activity. This phosphatase seems to be derived from the perichondral osteoblasts in which it appears. It is true that the body of the cartilage contains reactive cells, but the low activity of the matrix indicates that these reactive cells do not, within the period of observation, eject their phosphatase, as Bourne (1943) showed that similar cells do in cartilage formed in holes drilled in adult bone. Rather the resemblance between these positive cells of the long bones and the phosphatase-rich endochondral osteoblasts of the future

vertebral bodies suggests that the former are also of endochondral function, and are not concerned in the production of perichondral phosphatase.

The data as presented in Table III demonstrate that the state of differentiation of the cartilage, the quantity of phosphatase accumulated, and the amount of calcium phosphate deposited, are interrelated. In every bone examined, it was found that the concentration of phosphatase rises at the same rate as the cartilage hypertrophies, and that when the phosphatase-rich osteoid reaches a certain thickness, bone begins to be laid down; this is equally true in the tibia, where the entire process is extremely rapid, and in the proximal phalanges, which remain for two days in the protochondrial stage, and do not commence ossification until the eleventh day. In no case was any one condition found to be out of phase with the others. Fell and Robison (1934), investigating Robison and Rosenheim's (1934) postulated second mechanism of ossification, found that this mechanism, the ability of a tissue to deposit bone salts from a supersaturated solution, develops gradually during the period when bone is normally deposited. Evidently this development is paralleled by the accumulation of perichondral phosphatase.

Recently Horowitz (1942), using the histochemical technique, has shown that in fetal rat heads of thirteen days or more glycogen and phosphatase are simultaneously present only in tissues that ossify, and he has thus given fresh impetus to the view, originally suggested by Harris (1932), that glycogen plays a role in calcification, being possibly the source of the required phosphoric esters. Considering Glock's (1940) report that in rat bones taken a few days before birth glycogen is most concentrated at the primary center of ossification, it might be of interest to know whether in the very early material investigated in this study the accumulation of glycogen also parallels the cartilage-phosphatase-bone correlation which has been observed. It is worth noting that, according to Dalton (1937), glycogen first appears in the chick liver on the seventh day, increases slightly, and then decreases between the ninth and thirteenth days, the very period in which ossification is proceeding most actively. Moreover, alkaline phosphatase vanishes from the liver cells just before glycogen begins to be stored there.

I am glad to express my indebtedness to Doctor Viktor Hamburger for his stimulating interest in this work; and to Doctor H. B. Steinbach for advice and aid generously given.

Summary

1. Both acid and alkaline phosphatases are present in the unincubated blastoderm of the hen's egg, and in all embryonic tissues during the first two or three days of development. The concentration of alkaline phosphatase is much greater than that of acid phosphatase.

2. Phosphatase persists as long as a tissue remains undifferentiated. As differentiation proceeds, phosphatase in some cases disappears and in others accumulates in higher concentration than in the primitive phase. Alkaline phosphatase is more widespread than acid.

3. The changes in phosphatase distribution in the principal soft organs up to the eighth day, and the relation of alkaline phosphatase to bone deposition in the hind limb up to the eleventh day, are considered.

4. The effects of a variety of chemical agents on both phosphatases are reported.

5. The possible significance of phosphatases in the processes of embryogenesis is discussed.

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